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ScienceDirect

Genomics 90 (2007) 389–396

GENOMICS

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A humanized *IKBKAP* transgenic mouse models a tissue-specific human splicing defect

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Received 7 December 2006; accepted 30 May 2007
Available online 17 July 2007

Abstract

Familial dysautonomia (FD) is a severe hereditary sensory and autonomic neuropathy, and all patients with FD have a splice mutation in the *IKBKAP* gene. The FD splice mutation results in variable, tissue-specific skipping of exon 20 in *IKBKAP* mRNA, which leads to reduced IKAP protein levels. The development of therapies for FD will require suitable mouse models for preclinical studies. In this study, we report the generation and characterization of a mouse model carrying the complete human *IKBKAP* locus with the FD IVS20+6T→C splice mutation. We show that the mutant *IKBKAP* transgene is misspliced in this model in a tissue-specific manner that replicates the pattern seen in FD patient tissues. Creation of this humanized mouse is the first step toward development of a complex phenotypic model of FD. These transgenic mice are an ideal model system for testing the effectiveness of therapeutic agents that target the missplicing defect. Last, these mice will permit direct studies of tissue-specific splicing and the identification of regulatory factors that play a role in complex gene expression.

Keywords: Familial dysautonomia; mRNA splicing; Transgenic model

Familial dysautonomia (FD; Riley–Day syndrome, hereditary sensory and autonomic neuropathy type III, OMIM 223900) is a debilitating disorder that is due to the poor development, poor survival, and progressive degeneration of the sensory and autonomic nervous system [1,2]. The loss of neuronal function in FD has many repercussions, with patients having gastrointestinal dysfunction, abnormal respiratory responses to hypoxic and hypercarbic states, scoliosis, gastroesophageal reflux, vomiting crises, lack of overflow tears, inappropriate sweating, and postural hypotension. Despite recent advances in the management of FD, the disease is inevitably fatal, with only 50% of patients reaching the age of 40 [3]. The clinical features of FD are due to a striking, progressive depletion of unmyelinated sensory and autonomic neurons. Fetal development and postnatal maintenance of dorsal root ganglion neurons are abnormal, and slow progressive degeneration is evidenced by continued neuronal depletion with increasing age [1].

FD is a recessive genetic defect with a high carrier frequency in the Ashkenazi Jewish population that ranges from 1 in 32 [4] to as high as 1 in 18 in those of Polish descent [5]. Recent studies identified mutations in the gene *IKBKAP* as the cause of familial dysautonomia [6,7]. Three FD-causing mutations have been identified: an intronic noncoding point mutation, IVS20+6T→C (c. 2204+6T→C, NM_003640.2), which leads to variable skipping of exon 20, and two missense mutations, R696P (four patients) and P914L (one non-Jewish patient) [6–8]. Importantly, all FD patients identified to date possess at least one copy of the IVS20+6T→C mutation; 99.8% of patients are homozygous for this mutation, and five patients are compound heterozygotes with a missense mutation [6–8]. The result of the IVS20+6T→C mutation is a decrease in splicing efficiency that causes skipping of exon 20 in the *IKBKAP* message. This skipping event results in a frameshift and the introduction of a premature termination codon. All FD cells express both wild-type mRNA (WT) and mutant mRNA (MU) that lacks exon 20.

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doi:10.1016/j.ygeno.2007.05.012

The mutation thus weakens, but does not completely inactivate the 5′ splice site of exon 20. It is important to point out that FD is a recessive disease and there is no evidence that the mutant message is translated. We have previously shown that the mutant message is likely targeted for nonsense-mediated decay, and we can measure the relative amounts of WT:MU isoforms in FD cell lines and tissues [9]. This ratio varies between tissues, with the greatest amount of MU isoform produced in tissues from the central and peripheral nervous system [10]. Therefore, the pathogenic result of the *IKBKAP* splice mutation is a tissue-specific reduction in normal IKAP protein below a tolerable threshold. The neuronal phenotype of this disease suggests that the *IKBKAP* levels in specific neurons fall below the threshold required for normal development and maintenance. The IKAP protein is a subunit of the highly conserved Elongator complex, which is involved in transcriptional elongation [11]. RNA interference studies have shown that depletion of IKAP, and therefore Elongator, results in reduced transcription of a number of target genes via histone H3 hypoacetylation [12]. A number of these target genes play a role in cell motility, and FD patient cells were shown to be defective in standard cell migration assays. It is likely, therefore, that tissue-specific depletion of IKAP due to defective mRNA splicing leads to transcriptional dysregulation and impaired cell motility, which may be responsible for the neuropathology seen in FD.

The study of alternative splicing and of disease-causing splicing mutations has highlighted the importance of *cis*-acting control sequence elements that contribute to exon recognition and correct splicing, as reviewed in [13–15]. In addition to the well-characterized canonical sequences of the branch point and the 5′ and 3′ splice sites, lesser defined enhancer and silencer elements in both exonic and intronic sequences have also been widely reported. The regulation of splicing is a complicated, multilayered process, and despite recent progress in the field, the "splicing code" that dictates when an exon is skipped or included in the final transcript is still poorly understood. Detailed studies of *IKBKAP* missplicing have shown that *IKBKAP* exon 20 splicing regulation is no exception; exon 20 inclusion is delicately balanced and a variety of sequence elements contribute to the inclusion/exclusion decision [16,17].

The characterization of the *IKBKAP* splice defect in FD suggests that this disease is potentially treatable using strategies that improve exon 20 inclusion and subsequently increase the amount of normal IKAP protein in FD patients. Although FD is a developmental disorder, patients show continued neuronal degeneration throughout life, which leads to progressive ataxia and dementia in adulthood. An increase in IKAP at an early age might slow neuronal loss and reduce the symptoms that appear as patients mature. As part of a National Institute of Neurological Disorders and Stroke-sponsored drug screen, we previously identified the plant cytokinin kinetin as a potential therapeutic agent for FD [9]. We have shown that this small molecule dramatically improves *IKBKAP* exon 20 inclusion and consequently raises IKAP protein levels in cultured FD patient lymphoblast and fibroblast cell lines.

To examine the efficacy of kinetin and other potential therapeutic agents, it is desirable to have an accurate murine model for use in preclinical trials. In this study we describe the creation of an accurate *IKBKAP* splicing model. We created several transgenic mice expressing either human wild-type *IKBKAP* or FD (IVS20+6T→C) *IKBKAP* from a human BAC. We show that the presence of the FD mutation in the BAC causes missplicing of human *IKBKAP* in mice and that the efficiency of exon inclusion varies in a tissue-specific manner that closely models that seen in FD patients. Additionally, we show in tissue culture experiments that missplicing of human *IKBKAP* in mouse cells can be corrected by kinetin treatment, demonstrating conservation of cellular factors required for kinetin activity. The transgenic mouse model described in this study is an important and accurate system for preclinical testing of kinetin and other potential therapeutic agents aimed at improving *IKBKAP* splicing. Further, it demonstrates conservation of tissue-specific alternative splicing and provides a unique model system for unraveling this complex cellular process.

Results

Introduction of the IVS20+6T→C mutation into the human BAC

In our previous in vitro studies of *IKBKAP* splicing we showed that any number of slight sequence changes can alter splicing efficiency, highlighting the complexity of the control mechanism of exon 20 inclusion [17]. Splicing of mouse *Ikbkap* exon 20, in the context of both the mouse and the human sequences, is more efficient, and introduction of the FD mutation into the mouse *Ikbkap* gene does not result in exon skipping (data not shown), and therefore we pursued a transgenic rather than a knock-in model. To maximize the chance of accurately recapitulating the endogenous splicing mechanism, it is necessary to include in the transgene the complete genomic sequence including all intronic sequences and potential regulatory regions. We used the human BAC clone RP11-234B17, which contains the complete sequence of *IKBKAP* and approximately 20 and 80 kb of genomic sequence downstream and upstream, respectively (Fig. 1A). Also present in this BAC, located in close proximity to *IKBKAP,* are three other genes, *ACTL7B, ACTL7A,* and *C9orf6,* and the 3′ end of a fourth gene, *CTNNAL1*. To introduce the subtle FD point mutation into the BAC without altering any other sequence elements, we used a shuttle vector/recA-dependent homologous recombination method adapted from Lalioti and Heath [18]. This strategy involves two rounds of homologous recombination, the first involving positive selection for an integration event involving the BAC and a shuttle vector containing the homologous sequence bearing the mutation of interest. Fig. 1B shows a representation of the shuttle vector pKO3-KAN-FD, containing a 1.7-kb fragment of *IKBKAP* bearing the IVS20+6T→C mutation, which was created for this recombination strategy. The second step involves counterselection for a recombination event that resolves the shuttle vector/BAC co-integrant, removing the vector sequences and leaving the desired mutation within the BAC sequence in approximately 50% of the resolved constructs. The presence of the FD mutation in the

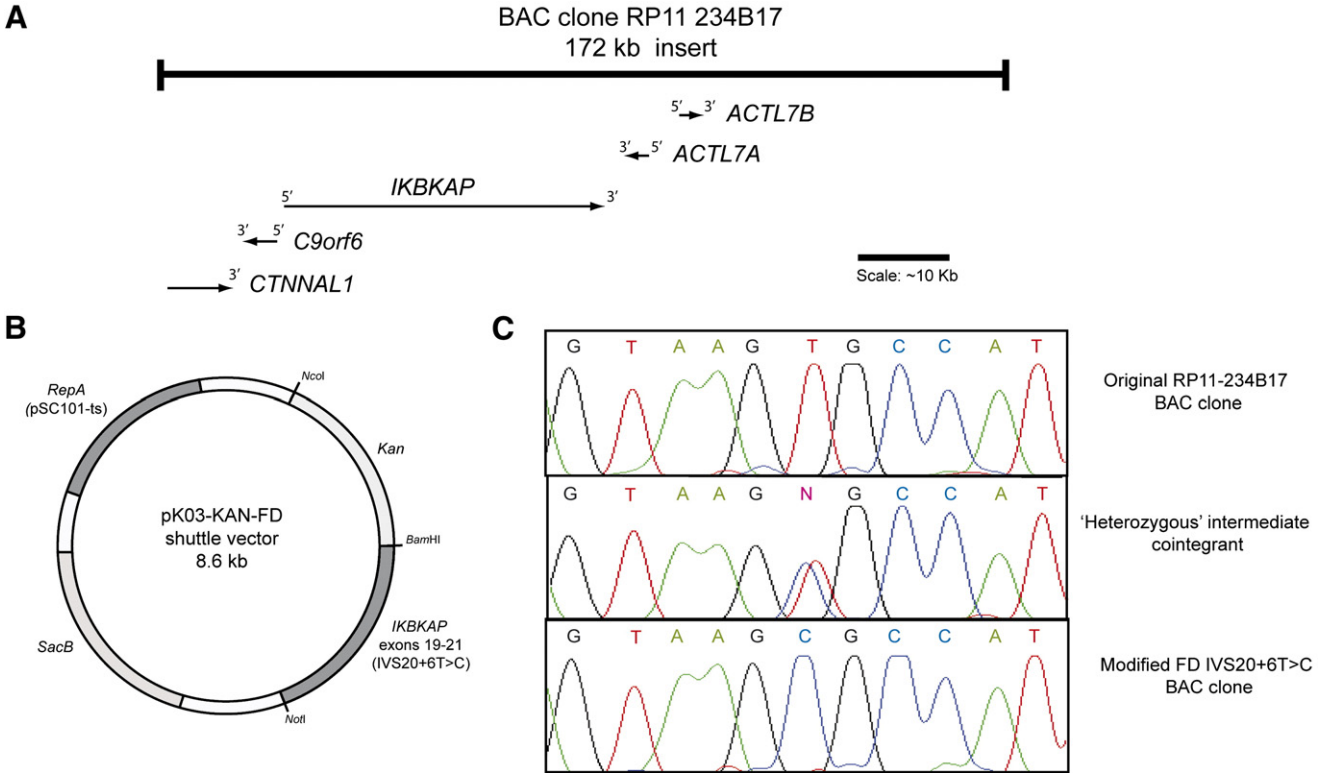

Fig. 1. (A) Schematic diagram representing the 172-kb RP11-234B17 BAC clone insert. The approximate sizes and locations of the complete genes *IKBKAP, ACTL7B, ACTL7A,* and *C9orf6,* and the 3′ end of *CTNNAL1,* are indicated. (B) Schematic representation of the shuttle vector pKO3-KAN-FD constructed for the homologous recombination strategy. The important features of pK03-KAN-FD are the 1767-bp genomic fragment of the *IKBKAP* gene including the FD IVS20+6T→C mutation; RepA (pSC101-ts), the temperature sensitive origin of replication; *SacB,* the levansucrase gene, a counterselectable marker; and *Kan,* a positive-selectable marker for kanamycin resistance. (C) Sequence analysis of exon 20 5′ splice site, in the unmodified RP11-234B17 clone, an intermediate co-integrant clone, and the modified FD BAC, being the IVS20+6T→C mutation. The intermediate co-integrant clone contains both the wild-type sequence and the FD mutation and hence appears "heterozygous" for T/C at position 6 of intron 20.

resolved BAC was confirmed by direct sequencing (Fig. 1C) and the integrity of the resolved BAC was confirmed by end sequencing with T7 and Sp6 primers and by restriction digest comparison to the original unaltered RP11-234B17 clone (data not shown).

Transient expression of wild-type and mutant FD IKBKAP in mouse N2A cells

Prior to generation of transgenic mice by microinjection of the BAC DNA, we tested for expression and splicing fidelity of both the wild-type and the FD BAC DNA. Purified DNA was transiently expressed in mouse N2A (neuroblastoma) cells. Seventy-two hours posttransfection, N2A cells were harvested and RNA was extracted. Using RT-PCR with verified human-specific primers, expression of *IKBKAP* was confirmed from both the wild-type and the FD BACs (Fig. 2A). Analysis of exon 20 splicing in *IKBKAP* expressed from the BAC DNA revealed that the wild-type BAC produced only the WT isoform, while the modified FD BAC produced both the WT and the MU spliced isoforms. This confirmed that the introduction of the FD IVS20+6T→C mutation in the human BAC leads to exon 20 skipping of *IKBKAP* when transiently expressed in mouse cells, modeling the effect of this mutation seen in FD patients.

Kinetin improves IKBKAP splicing in mouse N2A cells

We tested whether the potential FD therapeutic drug kinetin could improve exon 20 inclusion in *IKBKAP* transcripts expressed in mouse cells. N2A cells transiently expressing *IKBKAP* from the FD BAC were treated with 100 μM kinetin, and splice products were analyzed by RT-PCR (Fig. 2B). An improvement in exon 20 inclusion was observed in kinetin-treated samples, demonstrating that the cellular mechanism by which kinetin alters splicing is conserved between these two species. This finding is particularly encouraging as it suggests that mice expressing the human FD *IKBKAP* will be exploitable as models to test kinetin and other agents in preclinical animal trials.

Creation and characterization of humanized IKBKAP transgenic mice

After successfully detecting human *IKBKAP* expression from the BAC sequence in mouse cells, both the wild-type and the modified FD BAC constructs were used to generate transgenic animals by C57/BL6 oocyte microinjection. The initial microinjections yielded five FD and one wild-type ($Tg^{FD1–5}$ and Tg^{WT1}) founder animals, which were identified by PCR screening with primers specific to the human gene and con-

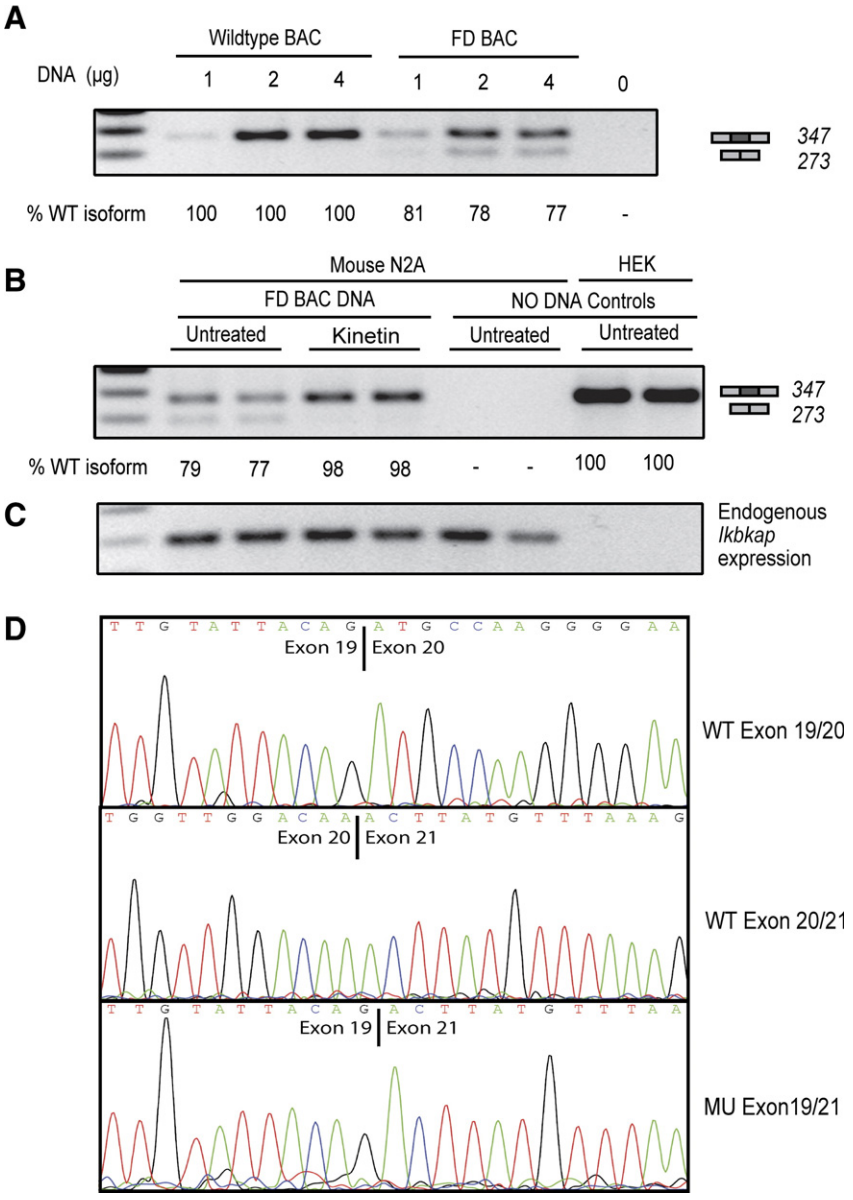

Fig. 2. (A) RT-PCR analysis on ethidium bromide-stained agarose gels of exon 20 splicing in mouse N2A cells transiently expressing human *IKBKAP* from increasing amounts (1–4 µg) of transfected wild-type and FD BAC DNA. The wild-type BAC expresses only the WT isoform, while the modified FD BAC expresses both the WT and the MU isoform. (B) Analysis of exon 20 splicing in kinetin-treated and untreated N2A cells expressing human *IKBKAP* from the FD BAC. An increase in the percentage of WT isoform is detectable with kinetin treatment. Also shown are untransfected mouse N2A and human HEK cells, demonstrating the specificity of the primers to amplify only human *IKBKAP*. (C) RT-PCR of N2A and HEK transfection samples as in (B) using mouse-specific *Ikbkap* primers. All transfections and treatments are shown in duplicate. (D) Sequence analysis of RT-PCR products of FD BAC showing presence of both wild-type and mutant spliced *IKBKAP* transcripts. Sequence at the junction of exons 19 and 20 (top) and exons 20 and 21 (middle) indicates the inclusion of exon 20 in the WT isoform. The bottom shows the sequence of the exon 19 and 21 junction confirming the absence of exon 20 in the mutant splice isoform.

firmed by Southern blotting. Approximate transgene copy number was estimated by both quantitative PCR analysis and Southern blotting against known quantities of BAC DNA and human genomic DNA [19] (Fig. 3). Evaluation of human *IKBKAP* expression and splicing in mice carrying the transgene was carried out by RT-PCR on total RNA extracted from heart, lung, liver, kidney, eye, and brain tissue. We detected expression of human *IKBKAP* in three of the six founder mice. The lack of expression in three of the founders is not unexpected and may be due to a number of factors, including copy number, insertion location, and disruption of transgene integrity, all of which can potentially affect BAC transgene expression [20]. Expression of human *IKBKAP* was detected in one wild-type *IKBKAP* transgenic animal (Tg^{WT1}) and two FD *IKBKAP* transgenic animals (Tg^{FD1}, Tg^{FD2}). Analysis of the human transcripts expressed from the wild-type *IKBKAP* transgene in Tg^{WT1} mice shows production of only the WT isoform (Fig. 4A), while transcripts expressed from the FD transgene in Tg^{FD1} and Tg^{FD2} showed production of both the WT and the MU (exon 20 skipped) isoforms. Importantly, the tissue-specific variability seen in the splicing patterns in these transgenic mice models what we previously observed in human FD patient

Mouse	Estimated Copy Number Southern Blot	DNA qPCR
Tg FD1	9	5
Tg FD2	2	7
Tg FD3	2	5*
Tg FD4	2	2
Tg FD5	33	28
Tg WT1	35	44

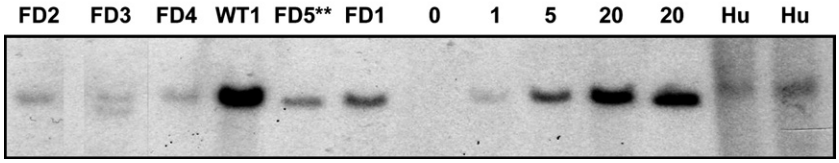

Fig. 3. (Top) Estimated copy number from six transgenic *IKBKAP* mice, calculated by Southern blotting and quantitative PCR of genomic DNA using human DNA (two copies) as a standard. *Multiple bands of varying sizes were present in the Southern blot, suggesting the integrity of the BAC was disrupted in this transgenic mouse. (Bottom) Southern blot analysis of transgenic mouse genomic DNA. Autoradiogram of BamHI-digested mouse genomic DNA (10 μg) separated on a 0.8% agarose gel and hybridized with a human-specific *IKBKAP* radiolabeled probe. The transgene number was estimated by comparing the intensity of the hybridized bands to the controls shown. **For Tg^{FD5}, only 1 μg of genomic DNA was used.

tissues [10]. Fig. 4B shows the relative amounts of WT:MU isoforms between an FD patient and the Tg^{FD1} mouse, for which matching tissue samples were available. The splicing efficiency is significantly decreased in the neuronal tissues compared to the nonneuronal tissues in both the FD patient tissue and the FD transgenic mouse tissue. The correspondence of the splicing pattern in heart, which displays more robust splicing, and lung, in which splicing is poor, is also notable. These results demonstrate that these transgenic animals will be extremely valuable for testing not only kinetin, but also other drugs that modify human splicing. Further, our demonstration that the factors governing tissue-specific splicing are conserved suggests that this mouse model will be useful for unraveling the complexities of mammalian splicing regulation.

Discussion

The availability of the human genome sequence allows the easy and rapid identification of large genomic BAC clones containing specific genes of interest that can be used for the production of transgenic mice for the study of both human disease and basic biological processes. Using large sequence BACs for transgenes is often preferable since genes are introduced in the context of their surrounding genomic sequences, including regulatory sequences and developmental or tissue-specific promoter sequences. In this study, use of a BAC was crucial to introduce all *cis*-acting splicing signals. A variety of homologous recombination techniques have been developed for accurate and subtle manipulation of large genomic clones, such as BACs, PACs, and YACs [18,21–23]. We found the introduction of a point mutation into a 172-kb BAC to be technically challenging; however, the need for more accurate and sophisticated murine models that recapitulate important aspects of human disease pathology will no doubt increase the use of these technologies.

In this paper we describe the generation of a transgenic mouse model that expresses human IVS20+6T→C *IKBKAP* and models the tissue-specific variability of *IKBKAP* exon 20 skipping seen in FD patients. The use of transient expression of the BAC construct in cultured mouse cells allowed us to confirm expression of human *IKBKAP* from the BAC construct prior to commencing with transgenic mouse production. Such a precautionary step is advisable to confirm that gene integrity has been maintained during the recombination procedures. Our cell culture experiments demonstrated not only exon 20 skipping as a result of the FD mutation, but also splicing improvement following kinetin treatment. Re-creation of these important attributes of the cellular FD phenotype in a mouse cell line indicated that the sequence elements that contribute to exon 20 missplicing are present and undisrupted in the modified FD BAC and that the mechanism of kinetin's action is conserved between the two species. Subsequent creation of the transgenic animals and demonstration that the tissue specificity of the splicing defect is also conserved highlight the general usefulness of this model for studies of tissue-specific splicing regulation and therapeutic development.

The original microinjections yielded six positive transgenic founder animals, and human *IKBKAP* expression was detected in three of these. The transgene copy number in the expressing and nonexpressing animals was variable, and high copy number did not necessarily correlate with detectable *IKBKAP* expression, suggesting that expression may be dependent on integration site as much as copy number. Host sequences surrounding the transgene can cause reduced expression or even transgene inactivation due to chromosomal positioning effects. Large genomic fragments such as YACs and BACs are usually insulated from such integration-dependent expression effects [20]; however, it is possible that some regulatory sequences were interrupted in our nonexpressing mice due to random linearization of the BAC during microinjection and integration.

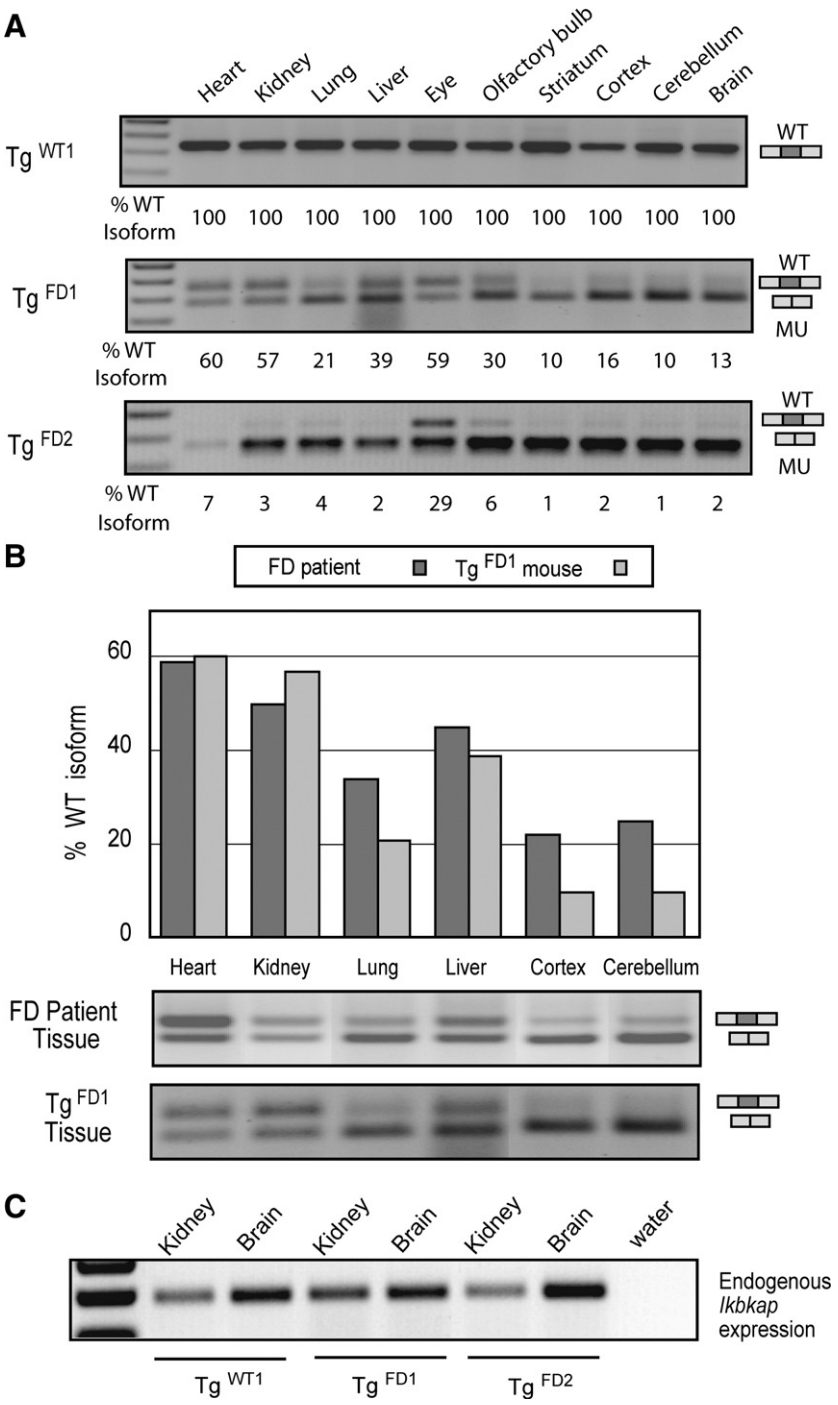

Fig. 4. (A) RT-PCR analysis of *IKBKAP* exon 20 splicing in multiple tissues from transgenic animals Tg^{WT1}, Tg^{FD1}, and Tg^{FD2}. Expression of the wild-type *IKBKAP* in multiple tissues produces only the WT isoform. Expression of FD *IKBKAP* in multiple tissues in Tg^{FD1} and Tg^{FD2} shows variable tissue-specific splicing of exon 20. (B) Comparison of *IKBKAP* exon 20 splicing in mouse and FD patient tissues. RT-PCR analysis of exon 20 splicing is shown for matching tissues samples from Tg^{FD1} mouse and an FD patient. Plotted in the bar chart is the relative % of WT isoform spliced in each tissue from both the mouse and the patient samples. A similar pattern of splicing is seen in both sample sets, with poorer exon 20 (% WT isoform) inclusion in neuronal tissues. The data for the human tissue samples are reproduced from previously published results [10]. (C) RT-PCR analysis of endogenous *Ikbkap* expression in kidney and brain from transgenic mice Tg^{WT1}, Tg^{FD1}, and Tg^{FD2}.

The conservation of missplicing in the transgenic mice carrying the FD mutation represents a major breakthrough in our efforts to develop a model system to test the efficacy of potential therapeutic agents. These animals can be used in preclinical trials of pharmacological agents previously shown to modify *IKBKAP* splicing, such as kinetin and EGCG [9,24]. These humanized transgenic mice also provide a beautiful model for studying tissue-specific alternative splicing. The similar splicing patterns seen in humans and mice demonstrate conservation of the mechanism responsible for tissue specificity. We hypothesized previously that the neuronal loss seen in FD is due to a drastic reduction of IKAP protein resulting from

low exon 20 inclusion [10]. This transgenic mouse model can be used to assess splicing patterns in individual cell types and identify genes that control tissue specificity. Furthermore, examination of transgene splicing in various mouse strains may lead to the identification of modifiers that influence human disease severity.

Creation of a true mouse model of FD is complicated because we must model a tissue-specific decrease in IKAP protein. The transgenic animals described in this study are a crucial step in the development of a phenotypic model of the human disease. Knockout of *ELP1/IKBKAP* has been shown to be lethal in *Drosophila melanogaster* [12], and preliminary results from our laboratory suggest that complete knockout of *Ikbkap* causes embryonic lethality in mice (data not shown). It will be of interest, therefore, to determine if the human wild-type *IKBKAP* transgene will be capable of rescuing lethality and ultimately if the FD transgene rescues lethality but leads to a mouse with a phenotype that resembles familial dysautonomia. The success of such a model system will certainly depend on overall expression levels of *IKBKAP* in the mouse, since high expression of even FD *IKBKAP* might be sufficient to overcome the absence of endogenous mouse *Ikbkap* expression. Thus, the creation of many *IKBKAP* transgenic lines with varying copy number and *IKBKAP* expression will be required to find an animal line expressing *IKBKAP* at levels functionally equivalent to those of endogenous mouse *Ikbkap*.

In summary, we have created several transgenic mice using a human BAC containing full-length *IKBKAP*, into which we inserted the IVS20+6T→C splicing mutation that is present in all cases of familial dysautonomia. The FD mutation leads to tissue-specific skipping of exon 20 in a pattern similar to that seen in FD patient tissue. The creation of these mice is crucial for testing potential therapeutics aimed at splicing modification, and they are an important step in the development of a phenotypic model of FD. Further, our demonstration that the factors governing tissue-specific splicing are conserved suggests that this mouse model will be useful for unraveling the complexities of mammalian splicing regulation.

Methods and materials

Constructs

The recombination shuttle vector pKO3-KAN-FD was constructed specifically to introduce the IVS20+6T→C mutation in the BAC clone RP11-234B17. The vector pKO3 (obtained from Professor George Church) was the basis for this construct and was modified in a number of ways: the 3′ region of the Cm^R gene was removed to disrupt chloramphenicol resistance; a kanamycin cassette (*kan*) derived from pACYC177 (NEB) was added to provide kanamycin resistance, and a 1.7-kb genomic fragment of the *IKBKAP* gene including the FD IVS20+6T→C mutation was subcloned from the *IKBKAP* 19-21 minigene construct [9]. The plasmid pDF25 was obtained from Professor David Sherratt and used unmodified.

Homologous recombination strategy

To introduce the point mutation in the BAC sequence we use a protocol based on that described by Lalioti and Heath [18]. Briefly, we prepared 50 μl of competent cells from 25 ml culture of DH10B cells containing BAC RP11-234B17 (grown at 37°C with 20 μg/ml chloramphenicol to an OD_{600} of 0.6) and washed them three times in ice-cold 10% glycerol. We cotransformed 750 ng pKO3-KAN-FD and 250 ng pDF25 into 50 μl of freshly prepared competent cells by electroporation (0.1-cm Bio-Rad Gene Pulsar cuvette, 1.8 kV, 25 μF, 200 ohms). We added 1 ml NZY+recovery medium and incubated the transformed cells for 1.5 h at 30°C. We then plated the cells on chloramphenicol and kanamycin plates and incubated them for 36 h at 30°C. We picked individual colonies into 1 ml of LB, of which we plated 100 μl on chloramphenicol and kanamycin plates and incubated them at 43°C overnight to select for co-integrants. The following day, we selected the larger colonies growing on a lawn of smaller colonies and prepared minipreps and glycerol stocks of these clones. We tested these potential co-integrant clones by sequencing to test for the presence of both wild-type and FD sequences. We selected co-integrants that appeared "heterozygous" T/C at position IVS20+6, for the counterselection phase. We then transformed 50 μl of freshly prepared co-integrant-containing competent cells with 250 ng of pDF25 by electroporation (0.1-cm Bio-Rad Gene Pulsar cuvette, 1.8 kV, 25 μF, 200 ohms). We added 1 ml NZY+recovery medium and incubated the cells for 1.5 h at 30°C. We then plated 100-μl aliquots onto chloramphenicol/sucrose plates and allowed co-integrants to resolve overnight at 43°C. We picked and streaked multiple colonies on chloramphenicol/sucrose plates and incubated overnight at 30°C to select for single colonies. We next selected the larger colonies and they were streaked onto both chloramphenicol/sucrose and kanamycin/chloramphenicol/sucrose plates and incubated overnight at 30°C. Only colonies that grew on chloramphenicol/sucrose and not on kanamycin/chloramphenicol/sucrose plates were considered to be resolved BACs. Resolved constructs were sequenced to confirm resolution and the presence or absence of the FD IVS20+6T→C mutation. The integrity of the resolved FD BAC was tested by end sequencing with T7 (5′-TAATACGACTCACTATAGGG) and Sp6 (5′-ATTTAGGTGACACTATAG) primers and by restriction digestion comparison with the original unmodified BAC RP11-234B17. Restriction digests were carried out with SmaI and XhoI and run on a 1% ethidium bromide-stained agarose gel.

BAC transfections and tissue culture

Mouse N2A cells were grown in 1:1 DMEM:HAMS, supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum. BAC DNA was purified using a Qiagen large construct kit, and 1–4 μg of BAC DNA was transfected into mouse N2A cells using Lipofectamine 2000 transfection reagent (Invitrogen). HEK cells were grown in DMEM supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin and 10% fetal bovine serum. Where noted, cells were incubated with of 100 μM kinetin solution (Sigma). Seventy-two hours posttransfection all cells were harvested and total RNA was extracted using Tri Reagent (MRC).

Generation of transgenic mice

BAC transformed bacterial cultures were grown at 30°C for 18 h. BAC DNA was isolated using anion-exchange columns (Nucleobond; BD Biosciences). Purified DNA was quantified by absorbance and analyzed by gel electrophoresis and then diluted into injection buffer [25] at 1 ng/μl. Transgenics were produced by standard methods [26], briefly: C57/BL6/N embryos were harvested from superovulated and mated females (Taconic and in-house). DNA solution was injected into a pronucleus of selected embryos that were then allowed to continue developing overnight. Those that advanced to the two-cell stage were surgically transferred to the oviducts of pseudopregnant outbred (Charles River or Taconic) recipient females. Resulting pups were weaned between 21 and 28 days after birth and tail biopsies were taken. Animals were treated in accordance with all relevant NIH guidelines.

Transgene analysis

Transgenic founders were identified by PCR using human-specific primers to *IKBKAP* intronic sequences TgProbe1F (5′-GCCATTGTACTGTTTGCGACT) and TgProbe1R (5′-TGAGTGTCACGATTCTTTCTGC). The transgene copy number in positive founder animals was determined by Southern blot using the same human-specific primers, TgProbe1F/TgProbe1R, to generate a 213-bp α-^{32}P-radiolabeled probe. Genomic DNA from potential founder mouse

tail biopsies was extracted using a Gentra Puregene genomic purification kit (Qiagen). A total of 10 μg of genomic DNA from each sample was digested with BamHI (NEB) overnight and separated on a 0.8% agarose gel. Copy number was estimated by comparing the intensity of the hybridized *IKBKAP* fragment in nontransgenic mice spiked with known quantities of the BAC clone with that of the same *IKBKAP* fragment present in the transgenic samples using ImageQuant TL software (Amersham Bioscience). Additionally copy number was also estimated by quantitative PCR analysis on the same genomic DNA samples using the Bio-Rad iCylcer, SYBR-Green supermix (Bio-Rad), and the primers TgProbe1F/TgProbe1R [19]. Copy number was estimated by comparing the amplification cycle thresholds attained in the transgenic mouse samples with human genomic samples (considered as two copies).

RT-PCR analysis of spliced isoforms

Reverse transcription was performed using 1 μg total RNA, oligo(dT) primer, and Superscript III reverse transcriptase (Invitrogen). PCR was performed using the cDNA equivalent of 100 ng of starting RNA in a 40-μl reaction, by use of Taq polymerase (Roche) and 30 amplification cycles (94°C for 30 s, 58°C for 30 s, 72°C for 30 s). Human-specific *IKBKAP* primers Hs-sp-Ex19F (5′-AGCAGCAATCATGTGTCCCA) and Hs-sp-Ex22R (5′-GTGACATCTTCTTCTTTCAA) were used to amplify human *IKBKAP*. Mouse-specific primers Mm-sp-Ex19F, 5′-AGTGGCAGTCATGAGGCCAG, and Mm-sp-Ex22R, 5′-GTGACATCTTCTTCCCTGAG, were used to amplify endogenous mouse *Ikbkap* transcripts. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The relative amounts of WT and MU *IKBKAP* spliced isoforms in a single PCR were determined using an Alpha 2000 image analyzer (Bio-Rad) and ImageQuant QL software (Amersham), using the integrated density value for each band as previously described [10], and the relative proportion of the WT isoform detected in a sample was calculated as a percentage. All PCR products were sequenced to confirm identity.

Acknowledgments

We thank all the FD patients and their families for their continued participation in our studies. We thank Dr. Felicia Axelrod of the Dysautonomia Treatment and Evaluation Center at New York University Medical School for her long-standing contributions to this work. We also thank George Church (Harvard Medical School) and David Sherratt (Oxford University) for generously providing us with plasmid constructs. This work was supported by grants from the Dysautonomia Foundation, Inc., the National Institute for Neurological Disorders and Stroke, and the Intramural Program of the NIMH.

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